

FRED Reports

THE FLUOROMETRIC DETERMINATION OF THE UPTAKE
AND RETENTION OF THE ANTIBIOTIC OXYTETRACYCLINE
IN SOCKEYE SALMON (*Oncorhynchus nerka*) FRY :
A QUANTITATIVE APPROACH TO TETRACYCLINE MARKING

BY

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ABSTRACT

Sockeye salmon (*Oncorhynchus nerka*) fry were fed oxytetracycline (OTC) medicated food to quantify the uptake and subsequent retention of antibiotic through the smolt stage. Our objective was to evaluate the use of OTC as an internal mark or tag. The widely practiced technique for qualitatively identifying OTC-fed fish under ultraviolet (UV) light is evaluated based on our quantitative fluorometric analyses of both total body burden and residualized OTC.

An OTC mark was detected in 100% of test fry following 29 days of feeding with OTC. The mean length and weight of fry at this time was 33 mm and 0.40 g. Additional feeding with OTC resulted in increased levels of both total body burden and residualized OTC, while control fish demonstrated neither a fluorescent mark, nor any change in fluorescence during the study period.

After the discontinuation of medicated food, experimental fry were found to retain a residualized mark into the smolt stage. The residualized mark stabilized at 0.64 μg OTC/fry in fish fed medicated food for 40 consecutive days (average weight of 1.0 g), and at 2.6 μg OTC/fry in fish fed for 61 consecutive days (average weight of 1.0 g). Thus, we were able to differentiate between wild fry and OTC-fed hatchery fry. More importantly, the ability to quantify OTC allowed us to differentiate between sublots of hatchery fry by generating different levels of residual OTC merely by altering feeding regimes.

INTRODUCTION

Returns of Pacific salmon (*Oncorhynchus* spp.) throughout the Pacific Northwest have been reduced by overfishing, construction of dams, and the degradation of spawning and rearing habitats (Hankin, 1982). In many areas, therefore, hatcheries have been constructed to artificially enhance salmon fisheries. Although hatchery production compensates for some depletion of the resource, it has been widely feared that hatchery production itself may lead to declines in returns of wild fish. Hankin (1982) cites two mechanisms whereby this might occur. First, interbreeding of wild and hatchery stocks could reduce the genetic fitness of the total population. Second, enhanced egg-to-smolt survival of hatchery fish, and the subsequent increased numbers of returning adults may enable hatchery stocks to withstand greater harvest pressures than natural stocks. Increased reliance on hatchery stocks might in turn promote fishery harvest rates that exceed sustainable limits for wild stocks, thus leading to further declines in abundance. Assessments of the survival rate from egg-to-smolt, and accurate discriminations between hatchery and wild stocks are therefore needed in order to evaluate the overall efficacy of hatchery programs.

Many hatcheries currently mark <30-mm fry with adipose (or other) fin clips, and/or coded microwire tags. Several factors render the above methods undesirable: 1) the regeneration of adipose fins is suspected, but the nature and rate of fin regeneration is at present unknown; 2) recognition of hatchery fin-clipped fry is complicated by natural fin loss; 3) only limited numbers of fish can be marked because of handling costs; and 4) increased stress on fry from displacement, handling, and mutilation during fin clipping results in a certain, but as yet unknown, amount of differential mortality.

Several authors have investigated the use of the antibiotic oxytetracycline (OTC) as a means of marking salmon (Weber and Ridgeway, 1962, 1967; Weber and Wahle, 1969; Odense and Logan, 1974; Wiltzius, 1980; Koenings and Lipton, 1983). The advantages of tetracycline marking lie in the possibility of inexpensively marking large groups of rearing fish by feeding them OTC-medicated food, while at the same time alleviating the physical mutilation and mortalities associated with fin-clipping and coded microwire tagging. Methods of detecting an OTC-positive mark, to date, have concentrated primarily on the identification under ultraviolet (UV) light of fluorescing OTC-calcium complexes incorporated in the skeletons of fish that were fed OTC-medicated food. For example, Weber and Ridgeway (1962, 1967) and Weber and Wahle (1969) successfully marked large groups of hatchery-reared sockeye salmon (*O. nerka*) fingerlings by feeding them oxytetracycline hydrochloride (2 g/kg body weight) for 3.5 consecutive days. Although fingerlings, as used in the above studies, are capable of rapidly incorporating far larger concentrations of OTC in their skeleton because of a larger proportion of calcified bone (the site of OTC residualization); the feasibility of OTC-retention in Alaska hatchery sockeye salmon fry which are considerably smaller is unknown. Wiltzius (1980) reported the successful marking of hatchery-reared kokanee by feeding fry (at an initial weight of 0.11 gm) OTC-medicated food (TM-50) for 7-11 days. Once again, marked fish were identified under UV light. These fry, however, had been

fed a medicated diet equivalent to 13.3 g OTC/kg feed, approximately three times the standard hatchery dosage prescribed for feed mixtures by the U. S. Food and Drug Administration.

Therefore, the objective of our study was to quantitatively examine the uptake and retention of OTC fed to hatchery-reared sockeye salmon fry in a standard medicated diet equivalent to 4.4 g OTC/kg feed. To our knowledge, this is the first study to be undertaken which uses a quantitative method to evaluate the use of the antibiotic oxytetracycline as an internal mark.

METHODS

Study Site Description

The sockeye salmon used in this study were taken from the Bear Creek stock of Tustumena Lake. Tustumena Lake is a large (29,449 ha), glacially-influenced lake located on the Kenai Peninsula in southcentral Alaska (Figure 1). Eggs were taken in August 1981, incubated, and the resulting fry reared at the Crooked Creek Hatchery near Kasilof, Alaska.

Experimental Fry

After emerging in the spring of 1982, fish were fed a diet of Oregon Moist Pellet (OMP) mash. In early June, approximately 5,000 fish were separated for study and placed in troughs in the Crooked Creek Hatchery. Fish began receiving OTC-medicated food on 3 June 1982. The medication used was Pfizer TM-50 (25 μ g OTC/0.2 mg TM-50), which was premixed with OMP at a rate of 4.5%--the highest production feeding rate prescribed by the U. S. Food and Drug Administration. On 13 July, 40 days after OTC-feeding had begun, a subgroup (A), was separated and placed on a diet of non-medicated OMP in order to examine the loss of OTC. On 3 August, after 61 days of OTC-feeding, the remaining fish (subgroup B) were taken off of the medication in order to compare the intensity and retention of the OTC mark in the two groups. On 8 August, all fish were transported to the U. S. Fish and Wildlife Service lab in Anchorage, where they were placed in circular tanks for rearing to smolts.

Control fish in this study are defined as natural sockeye fry with no previous exposure to OTC. These fish were used to demonstrate the natural background fluorescence of growing sockeye fry. We used control fish from Quartz Creek, a glacially-influenced stream that flows into the Kenai River approximately 160 km northeast of Kasilof. This was a desirable location from which to obtain control fry because the glacially-influenced water closely resembles that of Tustumena Lake, and the presence of a weir on the stream provided a source of sockeye fry throughout the study period.

Sampling Techniques

Test fry were periodically sub-sampled from the Crooked Creek Hatchery for laboratory analysis. In order to get a representative cross-section of the total population, the part of the trough out of which the fish were

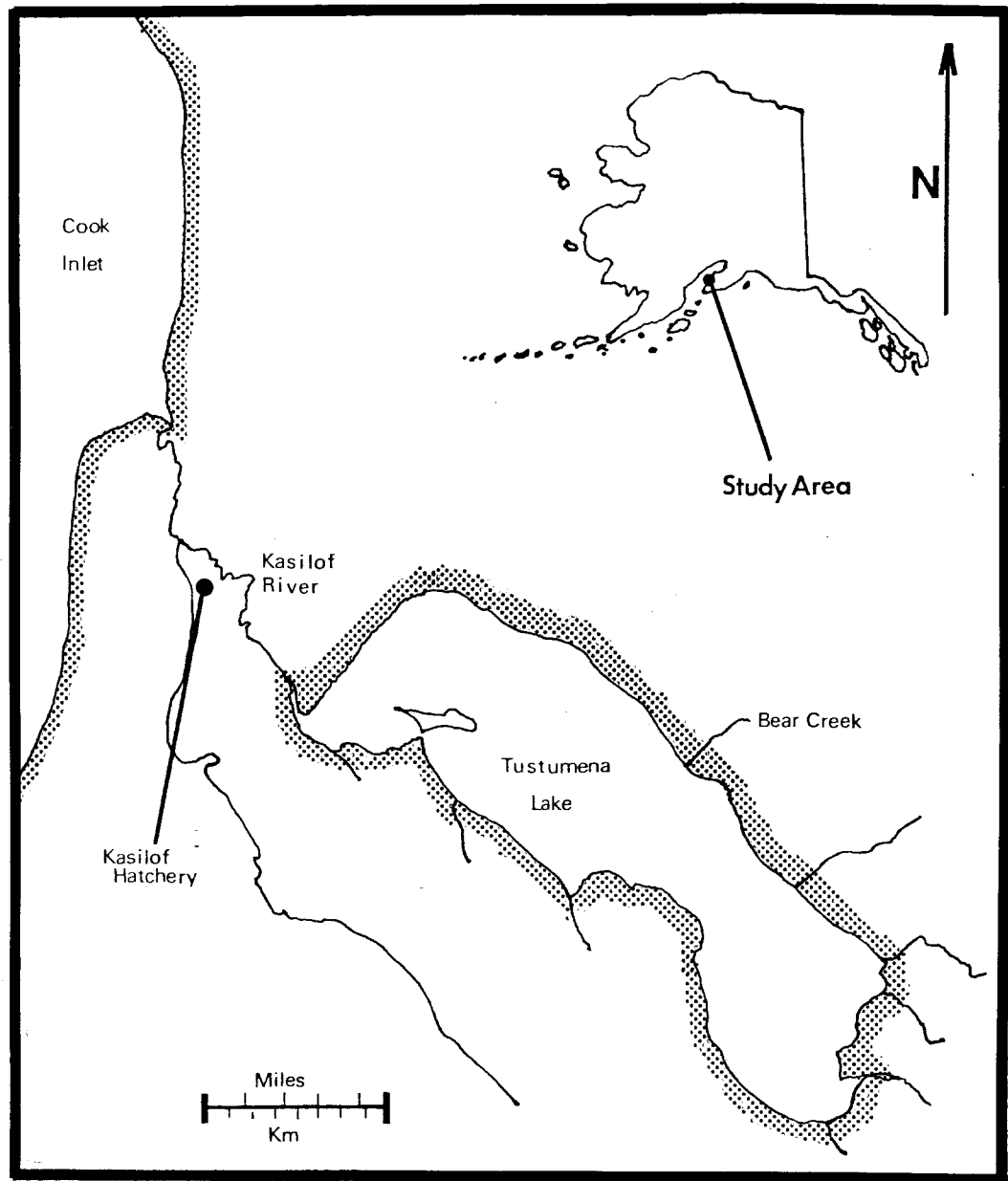


Figure 1. Location of Bear Creek in relation to Tustumena Lake and the State of Alaska Crooked Creek Hatchery.

sampled was constantly varied. Sampled fish were placed in plastic bags and transported fresh to the ADF&G limnology lab in Soldotna, Alaska for analysis.

Analysis of Fry Samples

Samples were analysed using the fluorometric technique of Koenings and Lipton (1983) at the ADF&G limnology lab in Soldotna, Alaska. The detection of residualized OTC was also performed using the technique of visual observation under ultraviolet (UV) light. Skeletons were dissected from fresh samples of test fry from Crooked Creek Hatchery and non-medicated control fry from Quartz Creek, briefly rinsed with deionized water, and placed under a Black-Ray 115 volt UV light. Both the exterior and cross sections of the skeleton were examined using a dissecting microscope in a dark room for evidence of fluorescent spots or rings. Fry exhibiting fluorescent spots or rings were called OTC-positive.

RESULTS

Storage of Fry Samples

Initially, fry samples were stored frozen until analyzed, as suggested by Gilliam and Argauer (1975) and Wiltzius (1980). Two effects were observed, however, that led to a reappraisal of this method of storage. The first effect was an increase in fluorescence values recovered on successive days of analysis from fry that were on medicated food at the time of sampling (Figure 2). This increase was caused by cellular rupture during freezing which allowed OTC to leak out of the gut into the flesh of the fry upon thawing. The effects of OTC leakage were found to be so pronounced that large increases were observed in values obtained between assay groups analyzed on the same day. That is, fish analyzed in the second assay group yielded fluorescence values 1.4 to 1.8 times higher than fish analyzed on the first assay (Table 1). In addition, mean fluorescence values from the second assay group consistently exceeded 95% confidence intervals computed for the first assay group. This indicated that the second group of fry during thawing were allowed to sit for a longer period before analysis, and hence OTC leaked through ruptured stomach cells into the flesh of the fry. The leakage of OTC was subsequently avoided by excising the digestive tract from all fish while they were still fresh. This also reduced the potential interference from naturally occurring fluorescent compounds primarily present in fat deposits around the stomach (Kohn, 1961).

Following adoption of the above procedure, it was found that the amount of OTC recovered from test fry was inversely related to the number of freeze/thaw cycles (Figure 3). Statistical analysis of the data (one-way analysis of variance for $p < 0.05$) showed a positive correlation between freezing and the loss or degradation of OTC. Thus, the use of freezing alone as a method of preserving fry samples was shown to be ineffective as it resulted in variable recoveries of OTC. However, because fry samples taken in the field had to be stored prior to laboratory analysis, the variable effects

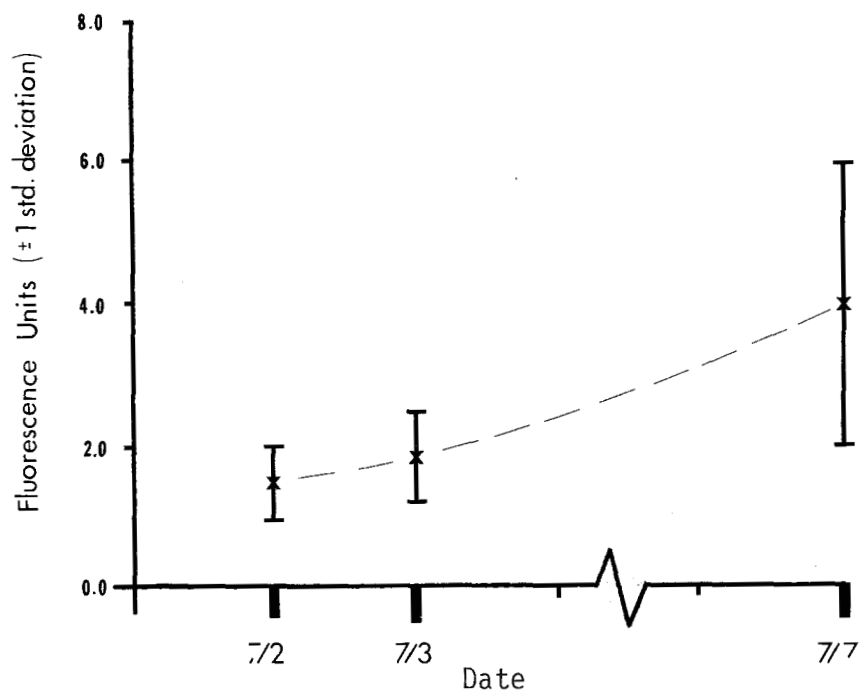


Figure 2. The increase in fluorescence found to occur within the same subplot of oxytetracycline-fed fry (with stomachs) stored in deionized water after three successive freeze/thaw cycles.

Table 1. Fluorescence values for assay groups of Crooked Creek Hatchery fry consecutively analyzed showing that mean fluorescence values of the second assay group fry tend to be outside the upper limits of the confidence intervals (95%) computed for group one fry.

Assay group	Date of analysis (1982)	Sample size	Fluorescence intensity [mean (μ)]	Standard deviation	Confidence interval (95%)
1	2 July	6	1.26	0.55	0.68-1.84
2		6	1.92	0.45	
1	6 July	10	1.65	0.51	1.21-2.01
2		10	2.23	0.72	
1	7 July	6	2.39	0.93	1.41-3.36
2		6	4.41	1.96	

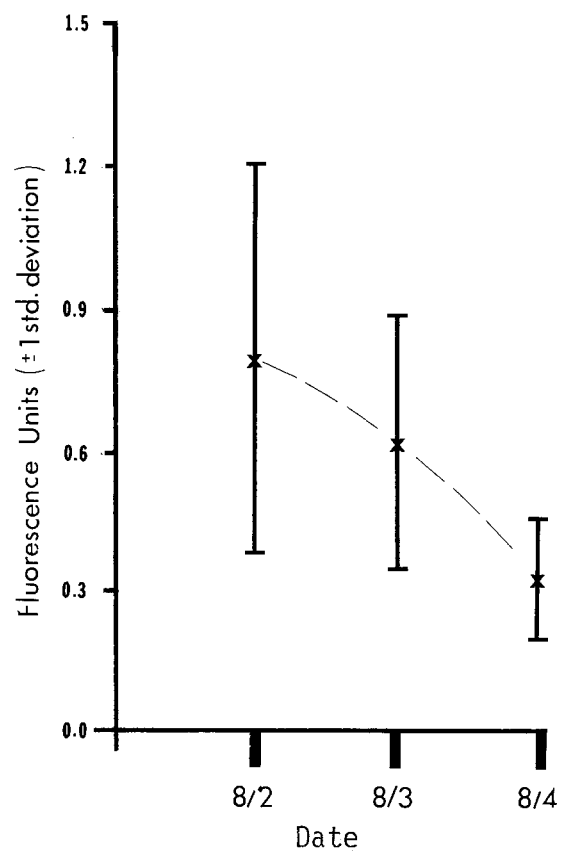


Figure 3. The decrease in fluorescence found to occur within the same subplot of oxytetracycline fed fry (stomachs removed) stored in deionized water after three successive freeze/thaw cycles.

of freezing on OTC made selection of a suitable method of storing fry a necessity.

Argauer and Gilliam (1974), and Ibsen et al. (1962) reported that OTC was stable when stored in solution with trichloroacetic acid (TCA). We therefore compared four methods of storing fry in 5% TCA (stomachs excised prior to storage): (1) refrigerating whole fry, (2) freezing whole fry, (3) refrigerating homogenized fry, and (4) freezing homogenized fry (Figure 4). Analyses of variance indicated that there was no significant difference ($p > 0.05$) in fluorescence values over time for any of the methods, except refrigerating homogenized fry in 5% TCA. However, even in this case, the absence of any discernable trend (either increasing or decreasing) indicates that the inconsistent value recovered on 8 September was a result of experimental error.

The highest recoveries were obtained by freezing homogenized fry. The mean amount of OTC recovered from test fry using this method was 5.6 μg OTC while 4.5 μg OTC was recovered from refrigerated homogenized fry, 4.0 μg OTC from whole frozen fry, and 3.3 μg OTC from refrigerated whole fry (Table 2). Freezing homogenized fry also produced the lowest variation within the subsample group (9% as compared to 18-25% for the other three methods). In general, we found that for both homogenized and whole fry samples, the recovery of OTC was approximately 1.2 times higher from fry stored frozen than from fry stored refrigerated, and 1.4 times higher from homogenized fry than from whole fry. This is probably a result of increased tissue surface area in contact with TCA in homogenized fry, and the increased extraction of OTC from cells ruptured during the freezing process. Since freezing homogenized fry in 5% TCA resulted in both higher and less variable recoveries of OTC, we used this method when storing fry and smolt samples for later laboratory analysis.

Growth of Experimental Fry

Throughout the study period, fry, fingerlings and smolts were fed a diet of Oregon Moist Pellet (OMP). Subgroup A fish were fed OTC-medicated OMP for 40 days, while subgroup B fish received medicated food for 61 days. Both subgroups of fish received non-medicated OMP after cessation of feeding the medicated diet. In spite of the different feeding regimes, we found no consistent size difference between subgroup A and subgroup B fish (Table 3). In addition, we found that the greatest rate of growth (by weight) occurred 20-30 days following the application of the medicated diet. In contrast, we found that as the rate of growth (by weight) decreased, the rate of growth (by length) increased, reaching its highest level 34-46 days after the initiation of OTC-feeding. Finally, by the summer of 1982 (9 August), the fingerlings had reached slightly over one third of the weight and nearly two-thirds of the length of the 19 July smolts (1983).

The Detection of Marked Fry

In our study of the uptake and the retention of OTC by fed fry, we first used visual observation accompanied by ultraviolet (UV) light. A definite yellow fluorescence (positive mark) was first observed in Crooked Creek

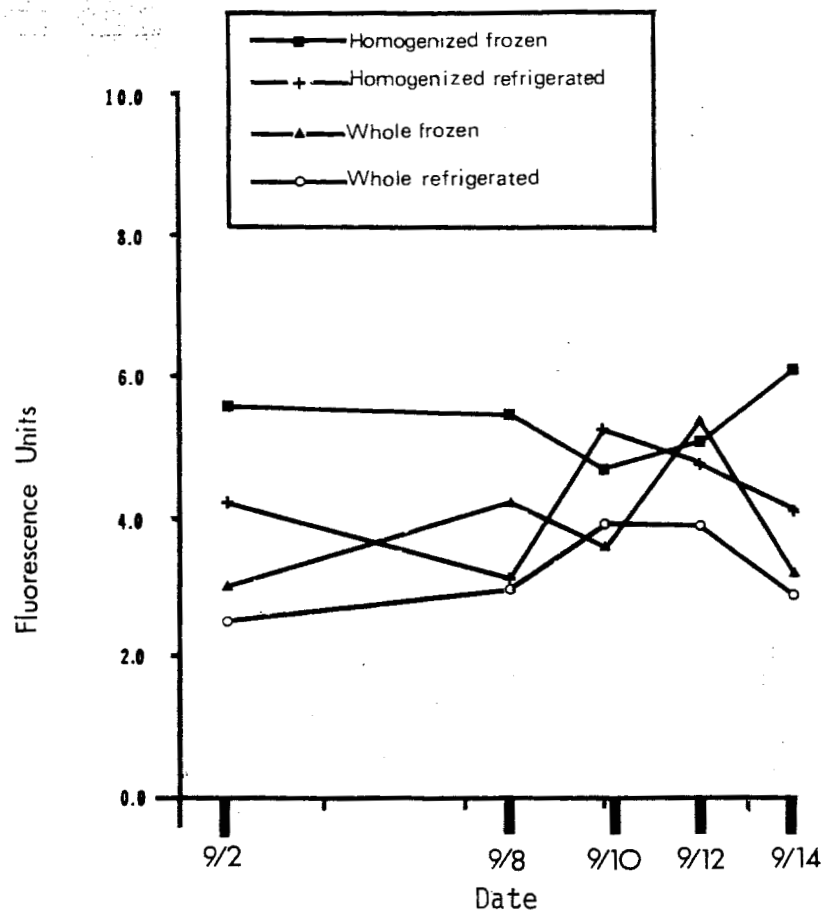


Figure 4. A comparison of the fluorescence intensity over time from OTC fed-fry (stomachs removed) stored in 5% trichloroacetic acid (TCA). Analysis of variance of the four storage techniques showed that oxytetracycline was effectively stabilized in each treatment by the presence of TCA.

Table 2. A comparison of the amount of oxytetracycline (OTC) recovered and the variability of recoveries from fry stored in 5% trichloroacetic acid using four preservation techniques.

Storage treatment	Mean oxytetracycline recovered (μg)	Standard deviation of sample means	Coefficient of variation (X 100)
Homogenized--refrigerated	4.5	0.83	18%
Homogenized--frozen	5.6	0.51	9%
Whole--refrigerated	3.3	0.60	18%
Whole--frozen	4.0	0.98	25%

Table 3. Crooked Creek Hatchery test fry used in the OTC feeding experiment showing date sampled, number of OTC feeding days, subgroup, mean weight, and mean length. The greatest rate of growth by weight occurred from t=20-30 days, while the maximal rate of growth by length occurred from t=34-46 days.

Date sampled	OTC feeding time (days)	Subgroup	Mean weight (g)	Mean length (mm)
<u>1982</u>				
3 June	0	AB	--	28
9 June	6	AB	0.18	--
14 June	11	AB	0.22	--
17 June	14	AB	0.22	--
21 June	18	AB	0.25	--
23 June	20	AB	0.24	31
28 June	25	AB	0.33	32
2 July	29	AB	0.40	33
4 July	31	AB	0.47	--
7 July	34	AB	0.48	34
12 July	39	AB	0.56	38
15 July	--	A	0.64	--
	42	B	0.69	--
19 July	--	A	0.68	42
	46	B	0.67	42
27 July	--	A	0.81	46
	54	B	0.77	44
29 July	--	A	--	46
	56	B	--	45
2 Aug	--	A	1.04	48
	60	B	1.00	46
5 Aug		A	1.12	49
		B	1.06	46
9 Aug		A	1.20	48
		B	1.35	48
<u>1983</u>				
27 April		A	3.13	73
		B	2.17	66
9 May		B	--	70
19 July		A	3.74	76
		B	3.48	76

Hatchery test fry 42 days after initiation of OTC feeding. At this time, 17% of the fry examined exhibited a fluorescing yellowish ring around their spine. Prior to this date, none of the fish examined (n=42) showed evidence of residualized fluorescing OTC. The percentage of fish marked gradually increased to 100% after 54 days (with only 40 days of medicated feeding followed by 14 days of a non-medicated diet) in subgroup A, compared to 100% marking rate in subgroup B fry achieved after 56 successive days on a medicated diet (Figure 5). An external, non-residualized mark was also observed, appearing as a yellowish sheen on the skin and gills of the fry.

To determine whether an OTC-mark could be fluorometrically detected in fry which had not shown any evidence of a mark when exposed to UV light, skeletons from UV-negative Crooked Creek Hatchery fry (OTC-treated) were analyzed using our chemical procedure. We found that 80% of the fry, which had been fed OTC for 31 days, were OTC-positive, containing an average OTC concentration of 0.66 μg . In contrast, no evidence of skeletal fluorescence was observed in any Quartz Creek control fry, although slightly fluorescent areas were found in the stomach of several of these fish. Similar naturally occurring fluorescent materials (e.g. lipids) were observed by Odense and Logan (1974) in Atlantic salmon (*Salmo salar*), and by Kohn (1961).

Our quantitative fluorometric technique was then used to analyze samples of both medicated and non-medicated fry, fingerlings, and smolts. We found the limit of detection of this technique to be 0.17 μg OTC/sample which was then used as a critical, or threshold level above which samples were termed OTC-positive (Figure 6) (Koenings and Lipton, 1983). The sample mean of Crooked Creek Hatchery test fry exceeded the critical level of 0.17 μg OTC 18 days after initiation of the feeding program, although only 56% of analyzed fish were found to contain a mark (Figure 6). However, after 29 days of OTC feeding, 100% of the fish were found to have a fluorescent mark with the sample mean at this time rising to 1.56 μg OTC/fry. The mark persisted in 100% of the fish sampled throughout the remainder of the feeding period.

In contrast, the mean value for all non-medicated control fry analyzed (n=216) was -0.18 μg OTC. In other words, the average fluorescence observed in non-medicated control fry was lower than the fluorescence of the reagent blanks (see Koenings and Lipton, 1983). Furthermore, we found that of the 216 control fry sampled, only 14 (6.5%) yielded fluorescence readings higher than the threshold level of 0.17 μg OTC/fry.

Total Body Burden of OTC in OTC-Treated Fry

The accumulation and subsequent residualization of OTC in fed fry was determined by holding an experimental group of 5000 fry in the Crooked Creek Hatchery following the release of nearly 17 million sockeye fry into Tustumena Lake. The experimental fry exhibited little detectable accumulation of OTC until after 18 days of feeding on the medicated diet (Figure 6). In general, the mean concentration of OTC (total body burden) in subsamples of fry increased as the time of medicated feeding increased, attaining a level of 1.83 μg of OTC/fry after 40 days of feeding. At this time, subgroup A fish were placed on a diet of non-medicated food, while subgroup B fish continued to receive OTC-medicated food.

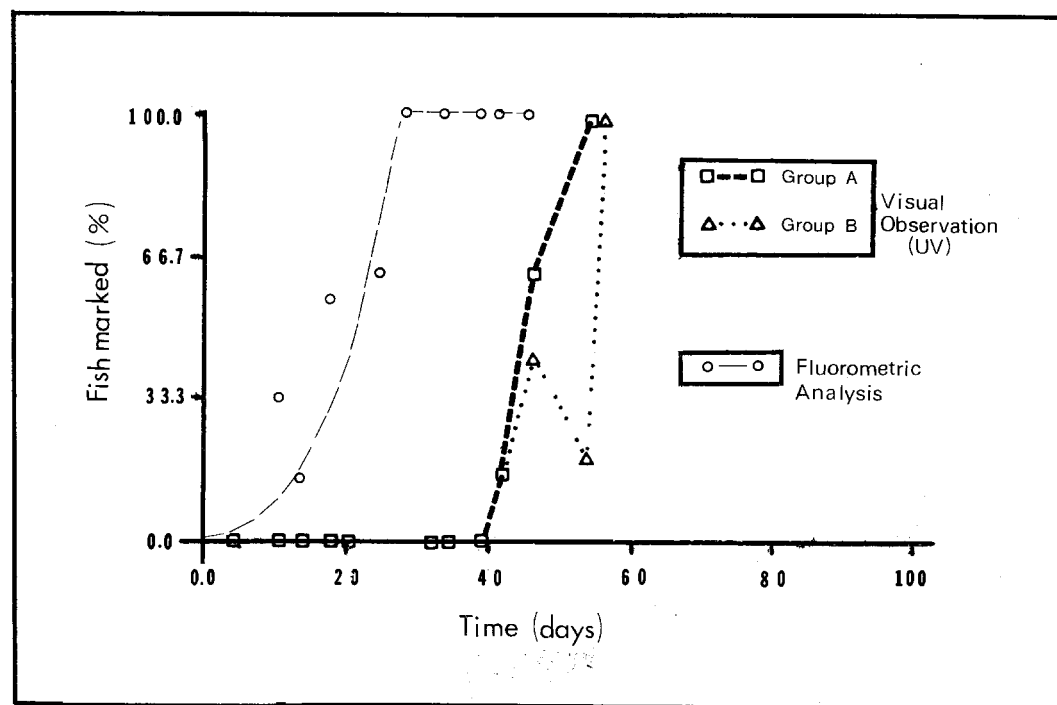


Figure 5. The percentage of OTC-fed fry found to be OTC-positive by both fluorometric analyses and by visual observation of skeletons under ultraviolet (UV) light.

After being taken off of the OTC-medicated food, subgroup A fry demonstrated an exponential decay in the total body burden of OTC, reaching a low of 0.49 μg of OTC/fry at $t=67$ days, which was 27 days after feeding with OTC had ceased. We considered, however, the residualized concentration of OTC (that which is held in the bone structure of the fry) to be represented by a mean level of 0.64 μg of OTC/fry recovered on the final four sampling dates in the summer of 1982 (i.e., $t=56$ through 67 days). This plateau was determined by an analysis of variance which showed that the levels of OTC contained within the fry during this 11-day period to be statistically ($p>0.05$) indistinguishable from one another. This definition was supported by our subsequent results from age 1.0 smolts. In the subgroup B fry, we found that as feeding with OTC was continued, the mean concentration of OTC recovered continued to rise. The highest recorded sample mean was 3.08 μg OTC/fry ($t=54$ days). This level of OTC decreased slightly to 2.5 μg OTC/fish at $t=60$ days, at which time feeding with OTC was discontinued. In contrast to subgroup A fry, subgroup B fry demonstrated a relatively small decrease in OTC concentration following cessation of the medicated diet.

On 8 August 1982, both groups of fish were transported to the U. S. Fish and Wildlife Service Laboratory in Anchorage for 9 months of rearing to the smolt stage. Because of time and distance delays involved in transporting samples from Anchorage to the limnology laboratory for analysis, we tested whether recovered levels of OTC differed between fresh (non-preserved) fish, and fish stored in 5% TCA. Our results using subgroup B smolts indicated that there was little difference between the two treatments (Figure 6), leading us to use fish stored frozen in 5% TCA (stomachs excised) for a majority of the smolt samples.

Finally, the experimental smolts were sampled in the spring of 1983 at the same time that smolts of the same brood-year were emigrating from Tustumena Lake. We continued to analyze both lots of experimental fish throughout the duration of the smolting period, and found that the levels of residualized OTC determined in the July-August fingerlings (1982) were present in the subsequent smolts produced in May-June (1983). That is, a residualized "mark" was retained unique to each lot of fish through the smolt stage (Figure 6).

OTC Mark Retention

Following the discontinuation of feeding with OTC-medicated food, a decrease in fluorescence was observed in both subgroups A and B fry. Subgroup A fry exhibited an average decrease in fluorescence of 66% over a 17-day period following cessation of the medication, stabilizing at an average level equivalent to 0.64 μg OTC/fry. Fry and fingerlings at this time weighed an average of 1.0 g. This mark was then retained in the fish through the winter and spring into the smolt stage. When analyzed in the spring and summer of 1983, fish (i.e., age 1.0 smolts) still had an average of 0.63 μg OTC/smolt, and averaged 3.13 g in weight and 75 mm in length. At this time, 91% of the fry tested were still found to demonstrate a detectable mark.

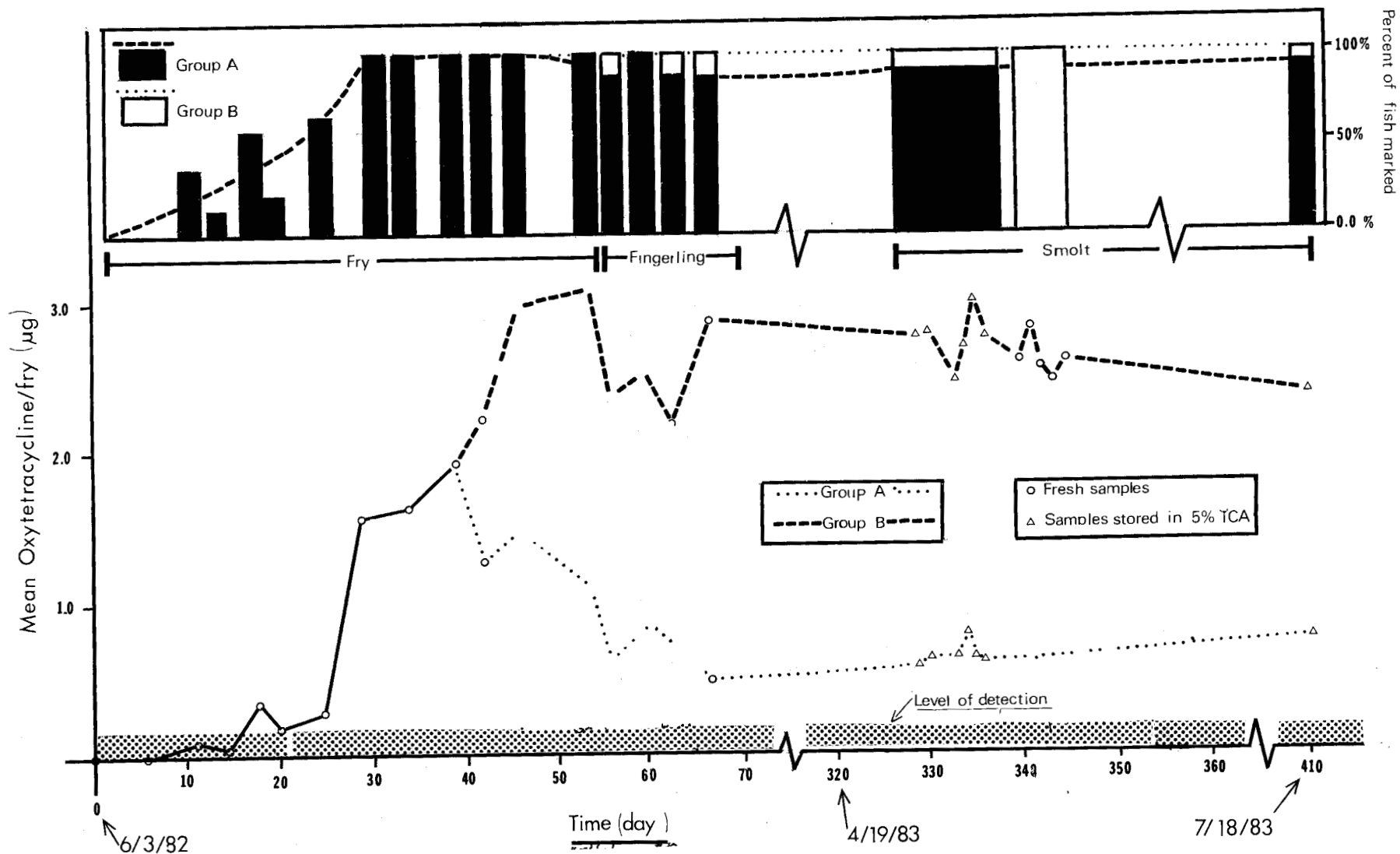


Figure 6. The concentraion of OTC recovered from the same brood-year sockeye salmon fry, fingerlings and smolts showing the detection limit of the method, the accumulation and loss of OTC from fry (group A and B) fed two regimes of medicated food, and the change in the percentage of marked fish throughout the study period.

In contrast to subgroup A fry, subgroup B fish exhibited only a slight decrease in fluorescence after feeding was discontinued at t=61 days, quickly stabilizing at an average level of 2.6 μg OTC/fry (average weight of subgroup B fish=1.0 g). This level of OTC-induced fluorescence was maintained through the winter and spring into the smolt stage, when 100% of the fish tested (averaging 2.2 g in weight and 66 mm in length) were still found to have a strong positive mark (2.58 μg OTC/smolt). By comparison, wild smolts of the same brood-year emigrating from Tustumena Lake during June of 1983 averaged 67 mm in length and 2.7 g in weight. As both subgroup A and B smolts were similar in size to wild smolts, we feel that the experimental fry and smolts served as useful analogs to wild stocks of the same age class and egg source.

DISCUSSION

The total body burden of OTC in salmon fed OTC-medicated food consists of both residualized and non-residualized antibiotic. Non-residualized fluorescing OTC is the amount of antibiotic complexed with calcium within the scales and/or skin of the fry. Since the calcium in the skin and scales has a faster turnover time than that in bone (Fleming 1974), the non-residualized OTC in the skin and scales will begin to disappear following cessation of OTC-treatment. That is, as the calcium within the skin and scales is used to meet the short term metabolic needs of the growing fry, the non-bone structures are flushed clear of OTC. In contrast, residualized OTC is the amount of the antibiotic that complexes with calcium that is laid into the bone structure. The residualized mark will thus consist of skeletonized OTC, and should therefore be retained for the life of the fish.

Evidence of this process can be clearly seen in the results generated in our OTC-feeding experiment. Subgroup A fish demonstrated an increase in fluorescence intensity during the period in which they received medicated food. This fluorescence resulted from both residualized and non-residualized OTC. Following discontinuation of the antibiotic, a decrease in fluorescence occurred resulting from the rapid loss of non-residualized OTC. This process of non-residualized OTC loss continued for approximately 17 days. At that time, fluorescence values began to stabilize at a level of approximately 0.63 μg OTC/fish. This concentration, representing the permanent residualized (skeletonized) OTC mark, was then maintained for a year, through the smolt stage, and should be retained for the life of the fish.

Subgroup B fry continued to receive medicated food for 3 weeks after subgroup A fry were placed on a non-medicated diet. Fluorescence intensity (resulting once again, from both residualized and non-residualized OTC) continued to increase with OTC-feeding. Following the cessation of feeding with OTC, little decrease in fluorescence intensity was observed, and fluorescence values stabilized at approximately 2.6 μg OTC/fingerling. This concentration, once again, represents the permanent residualized OTC that was retained by the fish through the smolt stage.

Both the large difference in the level at which the fluorescence intensity stabilized in subgroups A and B fry and the smaller amount of non-residualized OTC lost (total body burden - residualized OTC = non-residualized OTC) in subgroup B fry is related to the age in which the fry received medicated food. As fry grow, the rate of calcium deposition during formation of the skeleton increases (Fleming 1974). Therefore, the younger the fry, the greater the proportion of cartilage in the skeleton, and the smaller the total amount of calcium deposited. Since oxytetracycline is residualized as a calcium-complex, the greater the amount of calcium deposited, the greater the amount of OTC that will be residualized. Subgroup A fish were fed to an average weight of 0.40 g/fry, whereas subgroup B fry received food up to the size of 1.0 g/fry. Both the rate of calcium deposition and the total amount of calcified skeleton was therefore greater in subgroup B fry, resulting in a greater amount of OTC residualization. Furthermore, a smaller percentage of the total body burden of OTC in subgroup B fry was consequently composed of non-residualized OTC, so the relative amount of OTC lost after cessation of feeding was smaller.

The concentration of residualized OTC recovered from treated fry was found to be totally dependent on the length of OTC treatment, rather than being caused by a difference in fish biomass between the two treatment groups. That is, OTC induced fluorescence values stabilized in both subgroups A and B when fingerlings from the two subgroups were almost exactly the same size i.e., average weight of 1.0 g for both groups, yet the level of stable, residualized OTC recovered from the two subgroups was far greater in subgroup B fingerlings and smolts (0.64 μg OTC/individual in subgroup A compared to 2.6 μg OTC/individual in subgroup B). Thus, the observed difference in OTC level between the two subgroups reflected the additional 21 days that subgroup B was on OTC-medicated food. As such, we were able to generate a quantifiably distinguishable mark which could be used to differentiate between groups of fry, fingerlings, and smolts fed medicated food for different lengths of time. Our ability to distinguish between any two (or more) differently treated groups of OTC-positive fish far exceeds the scope of the visual technique.

A residualized mark in 100% of the Crooked Creek Hatchery test fry could not be detected using the technique of visual observation under UV light until 54 consecutive days of feeding, whereas, using the fluorometric procedure, a detectable mark was achieved in 100% of the fry after only 29 days. This clearly demonstrates the heavier OTC loading of rearing fry that would be required to achieve a detectable mark if visual observation techniques were used. In addition, fluorescing materials observed in the stomachs of known negative fry indicate the presence of naturally existing biological materials that could be interpreted as fluorescing OTC, thereby confusing the issue of mark detection. Finally, the visual detection of a 100% mark in subgroup A fish at 54 days, and in subgroup B fish at 56 days is not consistent with the quantitative data obtained for these fry samples. Subgroup B fish contained a far greater concentration of OTC than subgroup A fish, while a 100% mark was achieved 2 days earlier in subgroup A fish than in subgroup B. Also inconsistent with the quantitative results obtained, was the detection of a 100% mark in subgroup A fry at $t=54$ days,

when these fish were found to contain only 60% as much OTC as at t=40 days. Moreover, fluorometric analysis of fry skeletons perceived as OTC-negative under UV light showed them to be OTC positive.

The inconsistencies cited above centered on our ability to obtain quantitative data concerning the mechanism of the uptake and retention of OTC. That is, OTC may become incorporated into the cartilagenous "pre-skeleton" prior to becoming "residualized" within calcified bone. Furthermore, this residualization would be accompanied by a concentrating of the antibiotic in calcified rings as the skeleton is gradually laid down. It is this localized concentrating of the antibiotic within a calcium complex which enable visual detection of the "mark" under UV light. The fluorometric technique; however, can be used to detect OTC in fry before it is concentrated within calciferous rings. This would explain why a 100% mark rate using UV light was achieved in subgroups A and B only when the fish were approximately the same size, and points to a developmental process whereby OTC held within the cartilagenous skeleton was both residualized and concentrated during calcification of the vertebrae. This would also explain the visibility of the fluorescent mark at t=54 days in subgroup A fry, when the maximal concentration of total body burden of OTC ($1.83 \mu\text{g}$ OTC/fry) was detected at t=40 days i.e., OTC had not yet become sufficiently concentrated within the calcified skeleton at t=40 days for fluorescent rings to be visible. This narrows the period of calciferous residualization to be between t=40 and t=54 days, or at a fry size of 38-45 mm. Our finding that skeletons of fry (at t=31 days) perceived as OTC-negative under UV light, and were found to contain an average concentration of $0.66 \mu\text{g}$ of OTC indicates that the fluorometric technique is capable of detecting pre-residualized OTC within cartilagenous skeletal material before becoming concentrated as a calcium complex. Moreover, the fact that the mean concentration of OTC recovered from the skeletons was approximately equal to the mean level of permanent, residualized OTC found in fry and smolts may indicate that a certain loading level (determined by the net number of feeding days and the size of the fry) of OTC may be stored in the cartilagenous skeleton, later becoming residualized when calcium is laid down.

In addition, we found that the period of fastest growth in weight (t=20-30 days) corresponded to the period during which the total body burden of OTC was most rapidly increasing, while the period of fastest growth in length (t=34-46 days) occurred during the period of OTC residualization. This would explain the appearance of a visual, residualized mark (at t=54 and t=56 days) shortly following the period of greatest growth (by length).

Finally, as this method is capable of actually quantifying submicrogram levels of the antibiotic oxytetracycline in salmonid fry fed with OTC-medicated food, accurate discriminations can not only be made between wild and hatchery salmon, but also between sublots of hatchery fish fed to contain quantifiably different levels of residualized OTC. Thus, it would be possible to obtain accurate population estimates of sockeye fry within lakes using OTC marked hatchery fry. At the same time, this technique would alleviate the differential mortality stemming from fin clipping

and coded microwire tagging. The method could, in fact, be used to answer questions about both fin regeneration and the level of differential mortality associated with adipose fin clipping; currently the most common method used to identify salmon stocks.

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